IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: **Jon A. Wolff,**
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Group Art Unit: **1632**

Examiner: **Michael C. Wilson**

For: **Intravascular Delivery of Nucleic Acid**

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, Jon A. Wolff, hereby declare as follows:

1. I am an inventor of the captioned application.
2. I submit with this Declaration and Response further experimental material (attached) illustrating: delivery of polynucleotide encoding therapeutic proteins, VEGF and EPO, to limb skeletal muscle cells. The experiments were performed according to the methods provided in the Specification.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

J. A. Wolff 6/16/04

Jon A. Wolff Date

Increased vascularization following delivery of a therapeutic polynucleotide to primate limb.

DNA delivery was performed via brachial artery with blood flow blocked by a sphygmomanometer cuff proximately to the injection site. Left arm was transfected with VEGF, while right arm was transfected with EPO. The *Sartorious musle* from left leg was used as non-injected control. A male Rhesus monkey weighing 14 kg was used for these injections. The animal was anesthetized with Ketamin (10-15 mg/kg). A modified pediatric blood pressure cuff was positioned on the upper arm. The brachial artery was cannulated with a 4 F angiography catheter. The catheter was advanced so that the tip was positioned just below the blood pressure cuff. Prior to the injection, the blood pressure cuff was inflated so that the cuff pressure was at least 20 mmHg higher than the systolic blood pressure. After cuff inflation, papaverine (5mg in 30 ml of saline) was injected by hand (~8 to 10 seconds). After 5 min, the pDNA solution was delivered rapidly with a high volume injection system. For the EPO injection, 10 mg of pDNA was added to 170 ml of saline and injected at a rate of 6.8 ml per second. For the VEGF injection, 10 mg of pDNA was added to 150 ml of saline, and injected at a rate of 5.4 ml per second.

After 65 days, the animal was euthanized by overdose I.V. injection of pentobarbital Ketamin (10 mg/kg). The entire *Pronator quadratus* and *Pronator teres* muscles from both sides were immediately harvested and fixed for 3 day in 10% neutral buffered formalin (VWR, Cleveland, OH). After fixation, an identical grossing was performed for left and right muscles and slices across the longitudinal muscles were taken. Specimens were routinely processed and embedded into paraffin (Sherwood Medical, St. Louis, MO). Four microns sections were mounted onto precleaned slides, and stained with hematoxylin and eosin (Surgipath, Richmond, IL) for pathological evaluation. Sections were examined under Axioplan-2 microscope and pictures were taken with the aid of AxioCam digital camera (both from Carl Zeiss, Goettingen, Germany).

To evaluate the effect of VEGF plasmid delivery on cell composition in muscle tissue and neo-angiogenesis, we used monoclonal mouse anti-human CD31 antibody (DAKO Corporation, Carpinteria CA). The immunostaining was performed using a standard protocol for paraffin

sections. Briefly: four microns paraffin sections were deparaffinized and re-hydrated. Antigen retrieval was performed with DAKO Target Retrieval Solution (DAKO Corporation, Carpinteria CA) for 20 min at 97°C. To reduce non-specific binding the section were incubated in PBS containing 1% (wt/vol) BSA for 20 min at RT. Primary antibody 1:30 in PBS/BSA were applied for 30 min at RT. CD31 antibody were visualized with donkey anti-mouse Cy3-conjugated IgG, 1:400 (Jackson Immunoresearch Lab, West Grove PA) for 1 h at RT. ToPro-3 (Molecular Probes Inc.) was used for nuclei staining; 1:70,000 dilution incubated for 15 min at RT. Sections were mounted with Vectashield non-fluorescent mounting medium and examined under confocal Zeiss LSM 510 microscope (Carl Zeiss, Goettingen, Germany). Images were collected randomly under 400× magnification, each image representing 0.106 sq mm. Because muscle fibers and red blood cells have an autofluorescence in FITC channel we use 488 nm laser to visualize these structures.

Morphometry analysis. Coded mages were opened in Adobe Photoshop 5.5 having image size 7 × 7 inches in 1 × 7 inches window, and a grid with rulers was overlaid. The number of muscle fibers, CD31 positive cells and total nuclei was counted in all 7 image's strips consecutively, without any knowledge of experimental design. T-Test for Two-Sample Unequal Variances was used for statistical analysis.

Results: Microscopic evaluation did not reveal any notable pathology in either muscle regardless of the gene delivered. Also, neither muscle showed any notable presence of inflammatory cells, except of few macrophages. Necrosis of single muscle fibers was extremely rare in both, occupying negligible volume and was not associated with infiltration/vascularization. However, in muscles transfected with VEGF-165 plasmid, the interstitial cell and vascular density (observed in H&E-stained slides) was obviously increased (FIG. 4), as compare to EPO plasmid administered muscle (FIG. 4). Based on morphologic evaluation, these newly arrived interstitial cells we suggested to be endothelial and adventitial cells, smooth muscle cells, and fibroblasts. To evaluate participation of endothelial cells in this neo-morphogenesis, we have counted the number of CD31 positive cells in EPO and VEGF delivered *Pronator quadratus* muscles (FIG. 5). To assure that comparable specimens were analyzed in right and left muscles, the number of

muscle fibers was counted per area unit (0.106 sq mm). The VEGF and EPO administered muscles were not different in muscle fiber number (means 30.5 and 31.6). The number of CD31 positive cells however was significantly increased by 61.7% $p<0.001$ (means 53.2 vs 32.9).

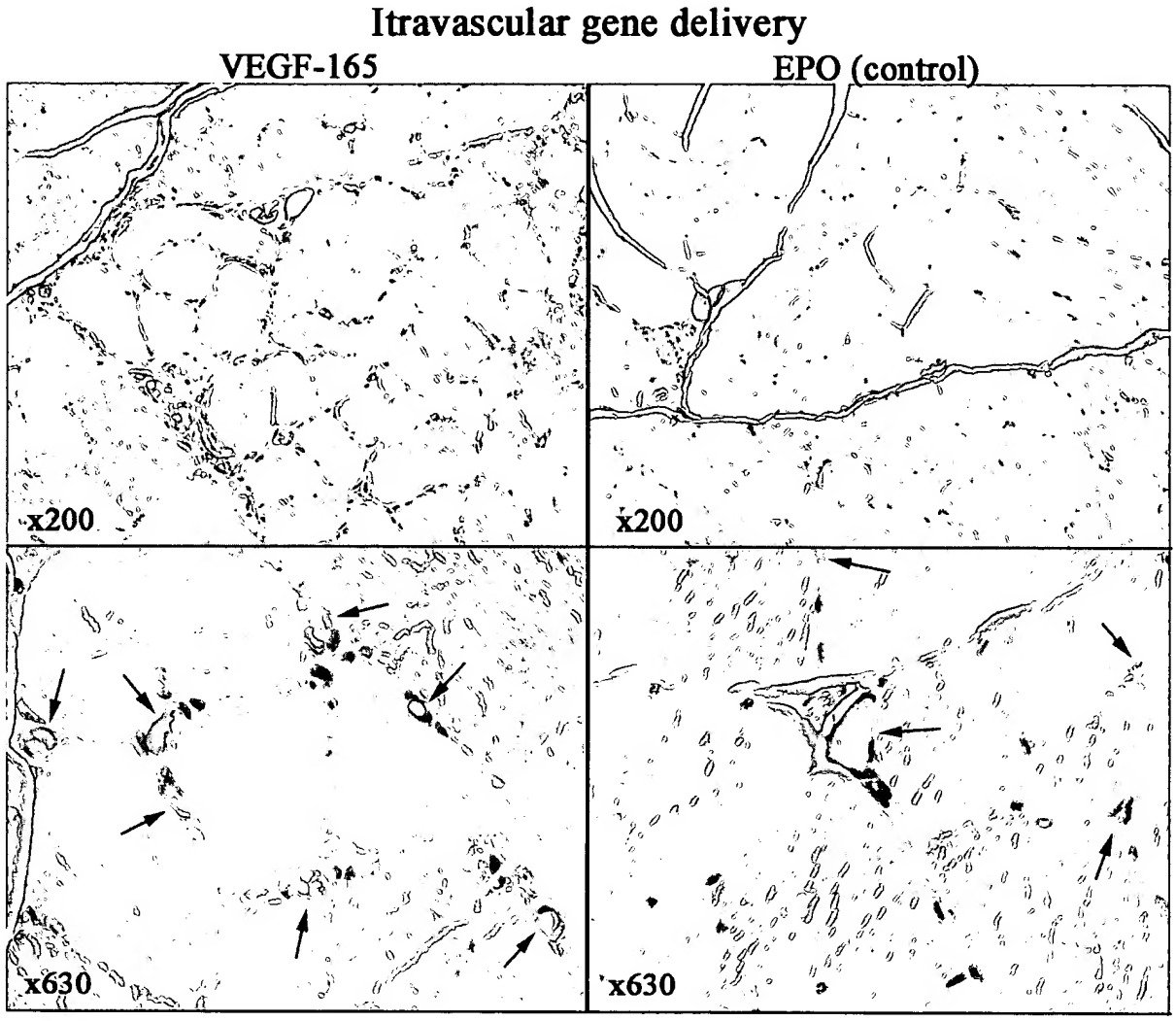

FIG. 4

Immunofluorescent staining (red) for endothelial cells (CD31)

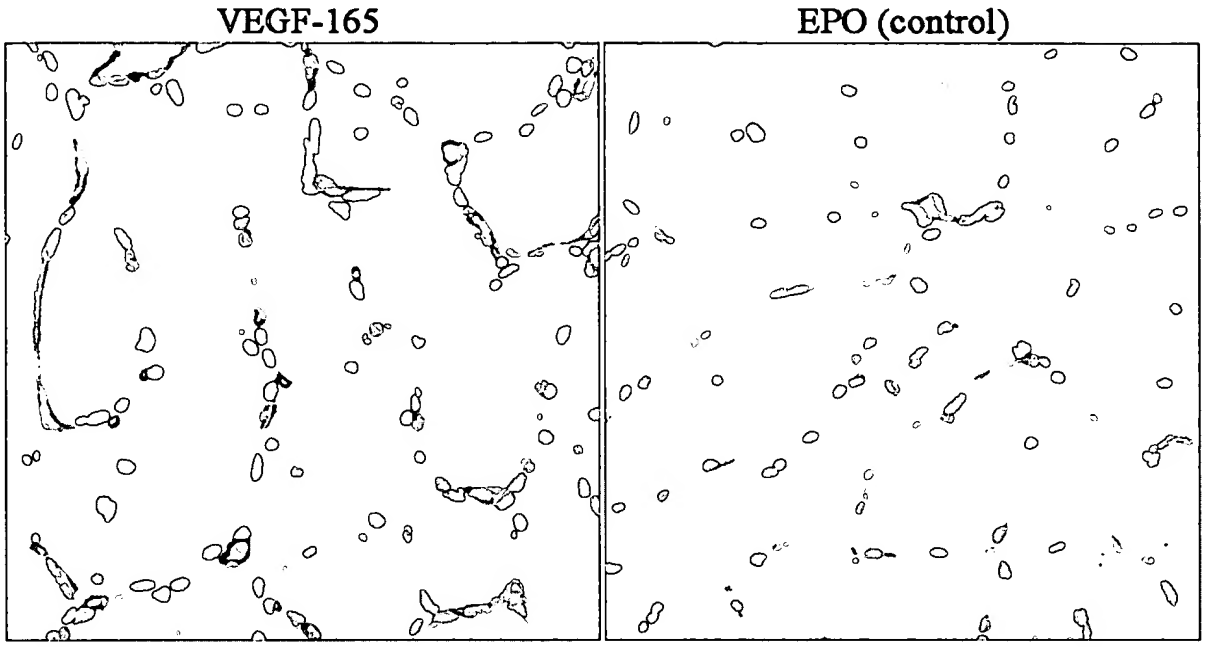

FIG. 5